Time to liquid culture positivity can substitute for colony counting on agar plates in early bactericidal activity studies of antituberculosis agents

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Abstract

The measurement of early bactericidal activity (EBA) is the first step in the clinical investigation of antituberculosis agents. EBA is determined by quantifying the viable sputum mycobacterial load on consecutive days of treatment. To investigate whether time to positivity (TTP) in mycobacterial liquid culture can substitute for colony forming unit (CFU) counting on agar plates we compared the error variation of TTP and CFU in 2115 pooled sputum samples collected overnight from 250 individuals included in five EBA studies. We found that the technical variation between duplicate laboratory measurements and the within-subject or day-to-day variation were similar for TTP (8.5% and 27.4% of total variation, respectively) and CFU (6.7% and 29.3% of total variation). The ability of the measurements to separate the EBA of 22 treatment arms was determined with group rank correlation of means and one-way analysis of variance. Except for the EBA over 0–2 days, individual and group EBAs measured with TTP and CFU were highly correlated. Treatment group means rank correlation coefficients were $r = 0.472$, $r = 0.910$ and $r = 0.818$, respectively, for EBA 0–2 days, EBA 0–7 days and EBA 0–14 days. Analysis of variance significantly favoured TTP over CFU for discrimination between groups with F values of 6.58 and 1.87, 7.77 and 4.58, and 8.71 and 3.56, respectively. We conclude that TTP is an acceptable alternative to CFU counting for the determination of the viable sputum mycobacterial load in EBA studies of up to 14 days duration.

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Background

In 1980 Jindani *et al.* [1] published details of an assessment of the early bactericidal activity (EBA) of antituberculosis agents by measurement of the decline in viable colony forming units (CFU) of *Mycobacterium tuberculosis* in sputum of patients with sputum-microscopy smear-positive pulmonary tuberculosis. Nearly all available antituberculosis agents were studied, either alone or in combination, over the first 14 days of therapy. A considerable amount of valuable information was generated by this study, which contributed to a better understanding of the actions of antituberculosis agents during early therapy. The determination of EBA is established as a first step in the clinical evaluation of a new antituberculosis agent. Involving a relatively small number of patients with smear-positive pulmonary tuberculosis, these studies confirm that the new agent is actually killing mycobacteria in sputum, enable the evaluation of its efficacy in relation to dosage and pharmacokinetics, and offer the opportunity for an early evaluation of toxicity.

Throughout the first 10–15 years of the exploration of EBA the quantification of *M. tuberculosis* in sputum depended on culture of sputum specimens on solid media and counting of viable CFU of *M. tuberculosis* at various dilutions expressed as logCFU [2]. While this methodology has proven its value over three decades, it is labour intensive and requires specialized laboratory facilities, and there is a relatively long lag before results become available. With the accelerating availability of new antituberculosis agents there is a need for a methodology that could also be used in less sophisticated communities where tuberculosis patients can be more readily recruited for appropriate studies, and that might measure the bactericidal activity of an agent in the same manner as sputum viable CFU counting.

An attractive alternative to enumeration of CFU for the quantification of *M. tuberculosis* is culture in liquid media and determination of the time to positivity (TTP), reflecting the detection of critical metabolic activity. Studies have demonstrated that liquid media are more sensitive than solid media for culture of mycobacteria from sputum and that TTP at diagnosis correlates with disease severity and treatment outcome in pulmonary tuberculosis patients [3–10]. To examine whether logCFU and TTP lead to the same conclusion, we examined the respective ability of both measurements to discriminate between 22 treatment arms in five recent EBA studies.

Methods and Materials

Sputum samples from five consecutive EBA studies were submitted to the MRC Centre for Molecular and Cellular Biology at the Faculty of Health Sciences, Stellenbosch University, Cape Town, between 2005 and 2010. The centre has abundant experience in EBA studies [2,11–16] and recruits patients from urban areas where the incidence of tuberculosis is estimated to exceed 1000/100 000 cases per year [17]. Consenting adults with smear-positive pulmonary TB were randomized to different daily drug dosage with experimental or positive control antituberculosis agents over 7 to 14 days. Sputum was collected overnight at regular intervals to monitor the fall of the viable bacterial load. The relevant ethical and regulatory bodies approved each study and every participant gave written informed consent. The results of three studies have been published to date [11,12,16].

Patients and sputum samples

Two hundred and fifty patients were recruited into 22 treatment arms with either experimental compounds (TMC207, OPC-67683, PA-824) or control regimens (H, R or HRZE). Median group size was 12 subjects (mean, 11.4; range, 6–16). Sputum was sampled at baseline before drug treatment and daily or every 2 days thereafter. Two baseline sputa were collected in three studies. In one study logCFU and TTP were measured only at baseline and at day 7. Duration of drug treatment was either 7 days (two studies) or 14 days (three studies). A total of 2371 samples were submitted, of which 2115 (89.2%) were available for this analysis. Due to various technical issues, such as lack of growth and contamination, no TTP was available from 141 sputa (5.9%) and no logCFU from 173 sputa (7.3%). There was no significant difference between the mean TTP and mean logCFU of samples with and without matched measurements (data not shown).

Sputum samples and processing

All sputum samples were submitted to the same laboratory for analysis using consistent protocols for collection, transport and processing during the whole study period. Specimens were homogenized by magnetic stirring for 30 min. A maximum volume of 10 mL of sample was added to an equal volume of 0.1% dithiothreitol (Sputasol; Oxoid, Cambridge, UK), mixed well and left for 20 min to obtain digested sputum for further processing.

For CFU determination, two series of five ten-fold dilutions (1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4} and 1×10^{-5}) of digested sputum were prepared in sterile Tween/saline. Selective Middlebrook 7H11 agar, enriched with Middlebrook OADC (Becton Dickinson, Johannesburg, South Africa), was prepared by adding Selectatab (Mast; Bootle, Merseyside, UK) containing polymixin B sulphate (200 units/mL), amphotericin B (10 mg/L), carbenicillin (50 mg/L) and trimethoprim (20 mg/L) and poured into two-compartment 90-mm Petri dishes. A volume of 0.1 mL of each dilution, including the neat sample, was inoculated in quadruplicate onto the selective agar. The agar plates were incubated for 3 weeks at 37°C without supplemental CO_2 and CFU counted at that dilution, where 20–200 CFU were visible for counting. Counts were averaged and corrected for dilution factors to result in a CFU count per ml sputum.

For culture in BACTEC MGIT 960, 5 mL digested sputum was mixed with an equal volume of 2% NaOH (Mycoprep; Becton Dickinson) in a 50-mL centrifuge tube and decontaminated for 15 min at room temperature. The mixture was neutralized by addition of M/15 sterile phosphate buffer (pH 6.8) up to the 45 mL mark. This mixture was centrifuged for 15 min at 3000 *g*. The supernatant was discarded and the pellet resuspended in M/15 sterile phosphate buffer (pH 6.8) to give a final volume of 2 mL. Two MGIT tubes were pre-

pared by adding OADC enrichment and PANTA (Becton Dickinson) containing polymyxin B (6000 units), trimethoprim (600 μg), amphotericin B (600 μg), azlocillin (600 μg) and nalidixic acid (2400 μg), and 0.5 mL of the resuspended pellet inoculated into each tube. The tubes were incubated at 37°C in the BACTEC MGIT 960 system, which automatically flags positive cultures. TTP was averaged and recorded in hours.

Statistics

We analysed the data in three steps. First, individual TTP and logCFU measurements were examined for overall correlation and total variation. Total variation was further examined for the proportions of purely technical variation and within-subject variation. Technical variation as the proportion due to purely technical factors could be estimated from duplicate measurements of the same specimens in the laboratory. Within-subject variation, a component of which is the technical variation, can also be described as the random biological or day-to-day variation. This component was estimated from consecutive baseline samples from the same subject and also indirectly from individual residuals in regression of logCFU and TTP on time in days. Within-subject variation, when compared to between-subject variation, essentially determines the precision of individual EBA measurements.

Second, we calculated the EBAs for each subject with logCFU and TTP and examined the correlation for the periods 0–2, 0–7 and 0–14 days. For the purpose of this study, EBA was defined by the fall in logCFU or by prolongation of TTP in hours. The efficacy of treatment is judged by the daily change in measurements in sputum from Day x to Day y:

- For logCFU:
  EBA(logCFU) = [logCFU(Day x)−logCFU(Day y)]/[$y-x$]
- For TTP:
  EBA(TTP) = [TTP(Day y)−TTP(Day x)]/[$y-x$]

Third, mean EBA(TTP) and mean EBA(logCFU) values were calculated for the treatment groups. The ability of EBA(TTP) and EBA(logCFU) to discriminate between groups was examined in two ways: (i) by mean group EBA rank correlation, and (ii) by comparison of between-treatment variation relative to within-treatment variation using one-way analysis of variance (ANOVA).

Pearson or Spearman rank correlations were employed where appropriate. Linear regression was performed with the method of least squares. Error variation is expressed as mean ± standard deviation (SD), or as F statistic when ANOVA is used. Higher F values represent higher between-group variation relative to within-group variation and hence better discrimination between groups. p values of <0.05 were accepted as significant. All calculations and graphics were created with 'R' software (http://www.R-project.org).

Results

Individual measurements

A correlation plot of all individual TTP and logCFU pairs shows crowding of points and visually moderate correlation (Fig. 1a). The scatter away from the general trend is largely due to error variation in logCFU and in TTP. The total variation expressed as overall SD for TTP and logCFU is 50.44 and 1.088, respectively. In accordance with the ten-fold dilution technique employed for CFU counting we grouped log-

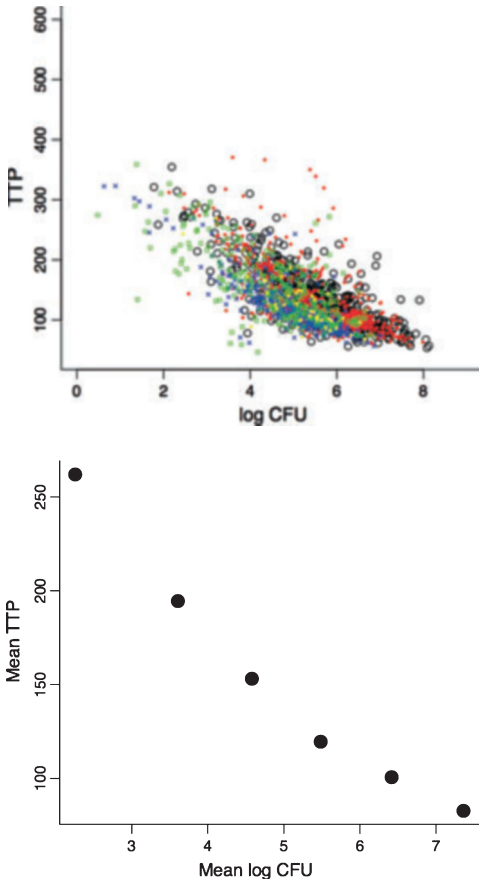

FIG. 1. Correlation of individual logCFU and TTP. (a) 2198 matched pairs of logCFU and TTP with a linear correlation coefficient of $r = -0.72$. There is considerable scatter, which is due to error variation of the measurements (SD for TTP, 50.44; for logCFU, 1.088). (b) Plot of mean TTP against mean logCFU values calculated by grouping logCFU in ranges <3, 3–4, 4–5, 5–6, 6–7, >7 and obtaining the means of logCFU and TTP of values corresponding to these logCFU ranges. The colour coding indicates different studies.

CFU in ranges <3, 3–4, 4–5, 5–6, 6–7, >7 (Fig. 1b). The correlation is clearly monotonic with a linear correlation coefficient of $r = -0.72$, but there is also a clear trend towards non-linearity in the range beyond 5 logCFU, hinting at an inherent threshold in the liquid culture system preventing a further decrease of TTP at higher logCFU levels.

The technical component of the SD for TTP was estimated as 4.287 from the differences between duplicate values used to create single TTP values. Single logCFU values were created by averaging from quadruplicates. In order to obtain a reasonably fair comparison of technical error we first created duplicates by taking the average of the two counts from the first agar bi-plate as the first duplicate and the average of the two counts from the second agar bi-plate as the second duplicate. The estimated technical SD from such duplicates was 0.0728. In relation to the variation between observations shown in Fig. 1, the mean technical SDs compared with the overall SDs give ratios of 4.287/50.44 = 0.085 for TTP and 0.0728/1.088 = 0.067 for logCFU. Proportions of 8.5% and 6.7% of the total SDs are small and of a similar order of magnitude, indicating that there is little to choose between TTP and logCFU with respect to the influence of technical error variation.

Within-subject variation of logCFU and TTP, a component of which is the technical variation, essentially determines the precision of individual EBA assessments. Measurements made on two successive days before the start of treatment were used to estimate within-subject (day-to-day) error variation directly from the differences between successive days (Table 1, studies 1–3). Within-subject variation was also estimated indirectly from residuals in regression of logCFU and TTP on time (days) for individual subjects. In order to include a similar number of different measurements such regressions were fitted on three studies from day 2 to the last day of study (Table 1, examples 4–6). In relation to the variation between observations shown in Fig. 1, the mean day-to-day error SDs compared with the overall SDs give ratios of 13.81/50.44 = 0.274 for TTP and 0.319/1.088 = 0.293 for logCFU. These proportions of 27.4% and 29.3% of the total SD are very close to each other, indicating, again, that there is nothing to choose between logCFU and TTP with respect to within-subject variation or technical error variation.

TABLE 1. Within-subject variation of logCFU and TTP

Successive drug-free days	logCFU, SD	TTP, SD
Example 1	0.400	13.34
Example 2	0.208	10.54
Example 3	0.288	7.58
Periods with drug treatment		
Example 4 (days 2–14)	0.444	19.13
Example 5 (days 2–14)	0.252	12.18
Example 6 (days 2–7)	0.323	20.08
Overall mean error variation, SD	**0.319**	**13.81**

Individual subject EBAs

Correlations between individual EBAs at days 0–2, 0–7 and 0–14 were all significant (p <0.0001) and improved from moderate to strong with increasing study duration ($r = 0.468$, $r = 0.649$ and $r = 0.741$, respectively). For EBA day 0–7, day 7 values were extrapolated where only day 6 and 8 were available, which resulted in the highest number of individual EBAs of the durations investigated (Fig. 2).

While the correlation of individual values, the error variation analysis and the individual EBA correlation give no indication that TTP is inferior to logCFU, the real test is how well TTP and logCFU discriminate between treatment arms. There are two issues: whether they assign the same ranks to treatments, and whether between-treatment variation relative to within-treatment variation is similar with the two measures.

Treatment group EBAs

Fig. 3 illustrates EBA(TTP) and EBA(logCFU) curves of 22 groups. Mean EBA(TTP) and mean EBA(logCFU) values were strongly correlated for all three periods measured (Table 2). It should be noted, however, that Pearson means correlations are strongly influenced by treatments that have much greater EBA values than the other treatments. This is particularly important for EBA day 0–2, for which the Spearman rank correlation coefficient is only 0.472, which, while still statistically significant (p 0.036), is much smaller than for EBA day 0–7 and EBA day 0–14.

Fig. 4 illustrates treatment means, ranks and separation between treatment means by displaying two-dimensional

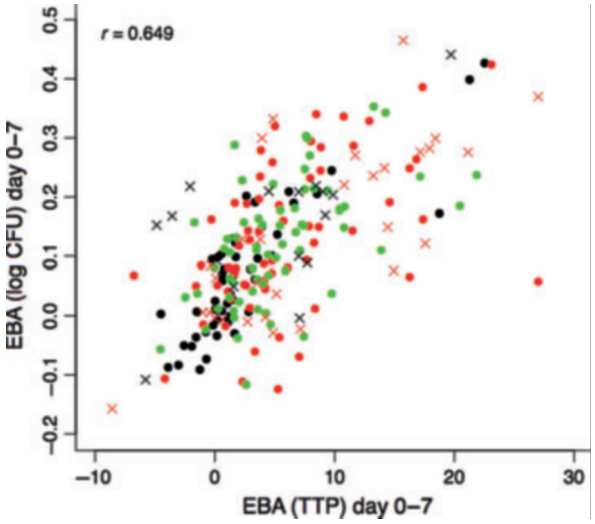

FIG. 2. Correlation of individual EBAs. This figure shows 224 individual EBA values for day 0–7 plotted against each other. EBA(logCFU) and EBA(TTP) are highly correlated with $r = 0.649$, p <0.0001. The colour coding indicates different studies.

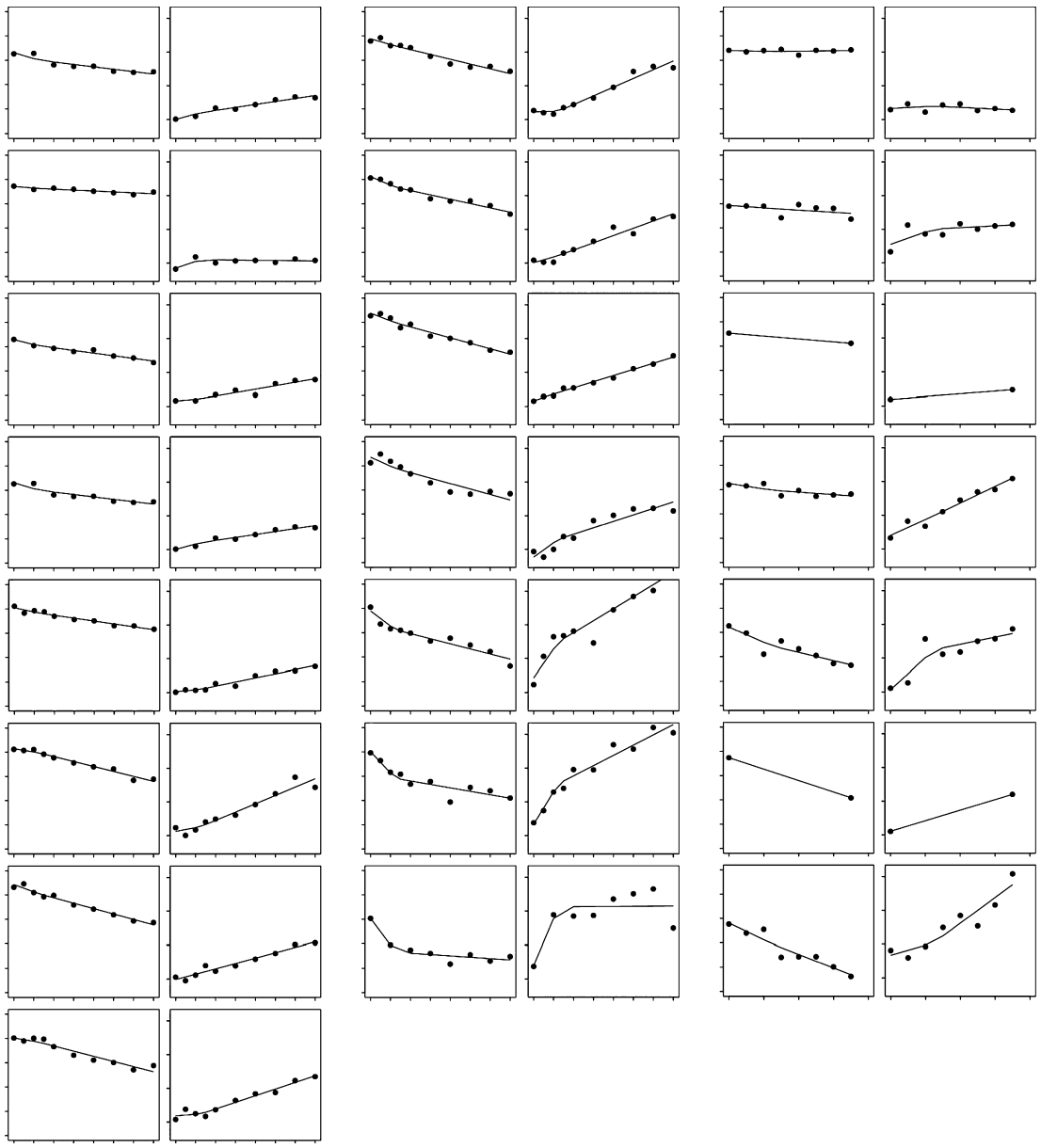

FIG. 3. Treatment group logCFU and TTP over time. Twenty-two treatment groups are shown. Each pair of graphs shows EBA(logCFU) on the left and EBA(TTP) on the right. The *x*-axis is scaled with 1 logCFU or with 50 h per tick mark, respectively, and the *y*-axis 2 days per tick mark. Fourteen-day groups are shown in the first and second columns and 7-day groups in the third column. Bilinear regression lines of logCFU and TTP on days are shown.

error bars. Non-overlap of error bars indicates discrimination on that axis. Inspection of Fig. 4 suggests, perhaps, that there is better separation of treatment means with longer study duration and there seems to be less overlap (i.e. greater separation between EBA(TTP) error bars than between EBA(logCFU) error bars). This impression is confirmed by the results of ANOVA calculations (Table 2). The between-treatment *F*-statistic for EBA(TTP) is considerably greater than the corresponding statistic for EBA(logCFU) and the difference is increasing with treatment duration. This means that the ratio of variability between groups and variability within groups is better with TTP than with logCFU and hence TTP discriminates better between EBA group means than logCFU.

Discussion and Conclusion

This study analysed a very large sample of paired logCFU and TTP measurements derived from consecutive EBA

TABLE 2. Mean EBA correlation coefficients and mean EBA one-way analysis of variance

		Group mean EBAs (Pearson)		Mean group EBA ranks (Spearman)		One-way ANOVA	
EBA days	*n*	*r*	p	*r*	p	*F* (CFU), p	*F* (TTP), p
0–2	20	0.898	<0.001	0.472	0.036	1.87, 0.019	6.58, <0.001
0–7	22	0.898	<0.001	0.910	<0.001	4.58, <0.001	7.77, <0.001
0–14	15	0.919	<0.001	0.818	<0.001	3.56, <0.001	8.71, <0.001

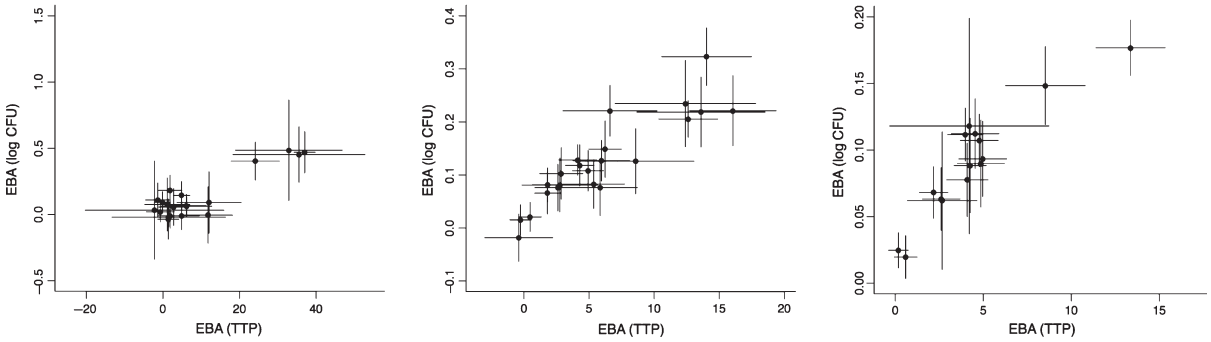

FIG. 4. Discrimination of group EBAs by logCFU and TTP. Treatment means and variation of EBA(logCFU) and EBA(TTP) in hours are calculated and plotted against each other. The error bars are shown with ±1.4 × SE limits, which are approximate 84% confidence limits. This has been calculated so that non-overlap of two of these intervals indicates a significant difference at the 95% level. Overlap or non-overlap of these error bars thus indicates how EBA(logCFU) and EBA(TTP) discriminate between treatment means for EBA day 0–2 (left), EBA day 0–7 (middle), and EBA day 0–14 (right).

studies, which included 16 experimental and six positive control arms. Apart from its size, the consistency of the method employed is the main strength of this report. All 250 subjects were recruited at a single urban centre, and all samples were analysed at the same laboratory. LogCFU and TTP measurements were highly correlated and showed similar technical and within-subject variation. Rank correlation of mean EBA and one-way ANOVA allowed the conclusion that for EBA studies of up to 14 days duration, activity measured by TTP in liquid media is a valid, if not better, alternative to activity measured by logCFU counted on solid media. The high degree of standardization and the superior technical accessibility could make TTP the preferred endpoint for clinical trials with antituberculosis agents in the future.

Liquid media have better sensitivity for growing mycobacteria from sputum than solid media. In treatment studies, this means that TTP will remain measurable for a longer period when sputum bacillary loads become low. This is not likely to be a significant advantage for 2-week EBA studies as long as experimental treatments are not considerably more effective than the current standard antituberculosis treatment. For a typical 8-week phase IIB study, however, TTP in liquid media could reduce the number of subjects with negative cultures at 8 weeks (i.e. measurements below detection level). Assuming that the error variation of TTP in liquid media remains similar to that of colonies counted on solid media up to 8 weeks, TTP could substantially increase the number of data points available for analysis in 8-week treatment trials, a duration of study that has been proposed to be sufficient to give an indication of sterilizing activity of regimens [18]. As a potential drawback, the greater sensitivity of liquid media could lead to more missing values due to contamination. This problem was not evident in the present dataset, but when sputum quality becomes poorer with increasing study duration contamination rates could increase, which could make the interpretation of serial TTP measurements difficult.

An interesting note is the non-linear trend of the relationship of TTP and logCFU observed in Fig. 1. From visual inspection it is plausible that an inherent threshold in the MGIT liquid culture system prevented TTP decreasing further when the number of inoculated mycobacteria should have allowed a lower TTP value. A possible reason for this observation is a bacterial lag phase induced by the sodium hydroxide-based decontamination procedure before for MGIT inoculation, which could delay the onset of metabolic activity independent of the actual number of bacteria inoculated. The affected area of the curve represents sputa with the highest bacterial loads, which are the basis of most individual EBA day 0–2 measurements. This can at least partly explain the

relatively poorer correlation of TTP and logCFU for EBA day 0–2. A potential solution for this problem is inoculation of a diluted sample for TTP, which would increase all TTP values and would allow TTP to spread over a wider range. As a disadvantage, diluted cultures might remain negative when the bacterial load becomes very low. This problem awaits resolution by performing comparative studies with TTP measurements derived from more than one dilution.

In conclusion, we demonstrated that TTP is an adequate and reliable alternative measurement for EBA studies of up to 14 days duration. This can have immediate consequences for site development, cost and speed of clinical antituberculosis drug evaluation.

Transparency Declaration

All authors declare no potential conflicts of interest.

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